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TSCA Confidential Business Information Center (7407M)  
EPA East – Room 6428 Attn: Section 8(e)  
U.S. Environmental Protection Agency  
1201 Constitution Avenue, NW  
Washington, DC 20004-3302

**REFERENCE: NO 8EHQ# Assigned As Yet**

Dear Sir/Madam:

As a follow-up to our previous electronic 8(e) submission dated, May 2, 2014, submitted for 4,4' (9H-fluoren-9-ylidene) bis(2-chloroaniline) identified by Chemical Abstracts Service Registry Number ("CASRN") 107934-68-9, please find enclosed the final report entitled "*Daphnia Magna*, Reproduction Test (Semi-Static).

This report **does not** contain confidential business information; therefore, a sanitized version is not necessary.

If you have any questions or comments please contact me at (973) 357-3375.

Sincerely,

Patricia Ann Vernon

Senior Manager, Global Product Regulatory Compliance/Toxicology

# FINAL REPORT

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## Study Title

### ***DAPHNIA MAGNA*, REPRODUCTION TEST WITH 4,4'-(9H-FLUOREN-9-YLIDENE)BIS(2-CHLOROANILINE) (SEMI-STATIC)**

## Author

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The Netherlands

## Laboratory Project Identification

**Project 503712  
Substance 205112/A**

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## 2. STATEMENT OF GLP COMPLIANCE

WIL Research Europe B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

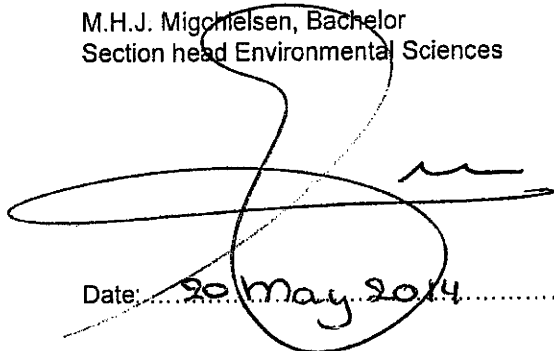
The Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (as revised in 1997) ENV/MC/CHEM (98) 17.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by WIL Research Europe.

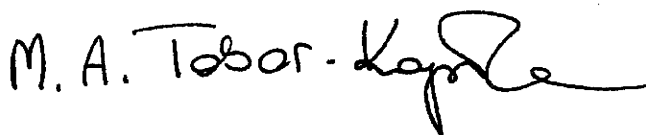
WIL Research Europe B.V.

M.H.J. Migchelsen, Bachelor  
Section head Environmental Sciences

M.A. Tobor-Kapton, PhD.  
Study Director



Date: 20 May 2014



Date: 21 May 2014

**3. QUALITY ASSURANCE STATEMENT**

WIL Research Europe B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the WIL Research Europe Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Project 503712

| Type of Inspections | Phase/Process                            | Start Inspection date | End Inspection date | Reporting date |
|---------------------|--|-----------------------|---------------------|----------------|
| Study               | Protocol                                 | 17-Oct-2013           | 17-Oct-2013         | 17-Oct-2013    |
|                     | Protocol Amendment 01                    | 04-Feb-2014           | 04-Feb-2014         | 04-Feb-2014    |
|                     | Test substance preparation               | 06-Feb-2014           | 06-Feb-2014         | 06-Feb-2014    |
|                     | Exposure                                 | 06-Feb-2014           | 06-Feb-2014         | 06-Feb-2014    |
|                     | Protocol Amendment 02                    | 20-Mar-2014           | 20-Mar-2014         | 20-Mar-2014    |
|                     | Report                                   | 01-May-2014           | 02-May-2014         | 02-May-2014    |
| Process             | <b>Analytical and physical chemistry</b> | 25-Nov-2013           | 04-Dec-2013         | 05-Dec-2013    |
|                     | Test Substance Handling                  |                       |                     |                |
|                     | Observations/Measurements                |                       |                     |                |
|                     | <b>Analytical and physical chemistry</b> | 10-Feb-2014           | 20-Feb-2014         | 27-Feb-2014    |
|                     | Test Substance Handling                  |                       |                     |                |
|                     | Exposure                                 |                       |                     |                |
|                     | <b>Environmental Toxicology</b>          | 17-Feb-2014           | 25-Feb-2014         | 25-Feb-2014    |
|                     | Test Substance Handling                  |                       |                     |                |
|                     | Exposure                                 |                       |                     |                |
|                     | Observations/Measurements                |                       |                     |                |

The review of the final report was completed on the date of signing this QA statement.

WIL Research Europe B.V.

Quality Assurance

*ANISEN MORGAN, Quality Assurance Auditor*

*A. Morgan*

Date: *21 May 2014*

#### 4. SUMMARY

*Daphnia magna*, 21-day reproduction study with 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline).

The study procedures described in this report were based on the OECD guidelines for Testing of Chemicals: Guideline No. 211, 2012. In addition, the procedures were designed to meet the test methods and validity criteria of the ISO International Standard 10706, 2000, the Commission Regulation (EC) No 440/2008 Part C.20, 2008 and the OECD guidance document number 23, 2000.

The batch of 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) tested was a white to off-white powder with a purity of 97.3% and the substance was not completely soluble in test medium at the loading rates initially prepared.

A Water Soluble Fraction was prepared at a loading rate of 5.0 mg/l and used as the highest concentration. Lower test concentrations were prepared by diluting the highest concentration in test medium. The final test solutions were all clear and colourless.

The reproduction test was performed in a semi-static system, included 10 vessels per test concentration and 20 vessels for an untreated control group. Each of the vessels contained one neonate (<24h old) *Daphnia magna* in 50 ml test medium. Daphnids were exposed to solutions containing 0.16, 0.80, 4.0, 20 and 100% of the WSF prepared at 5.0 mg 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) per litre.

The study duration was 21 days and the test solutions were renewed every 48 hours. The daphnids were fed on a daily basis with a *Chlorella pyrenoidosa* suspension. Every workday the condition of the parental daphnids was recorded, during the reproduction phase the number of living offspring, immobile young and appearance of unhatched (aborted) eggs was recorded. At the end of the test the lengths of the surviving parental daphnids were measured.

During the study samples for analyses were taken at the beginning and the end of four intervals of 48 hours.

Calculated average exposure concentrations corresponded to 6.2, 6.8, 14, 83 and 550 ng/l in solutions containing 0.16, 0.80, 4.0, 20 and 100% of the WSF prepared at 5.0 mg/l, respectively.

The study met the acceptability criteria prescribed by the protocol and was considered valid.

Mortality of parental daphnids observed in test concentrations was not statistically different from the control treatment.

The average cumulative number of young per female in the control after 21 days was 84. Significant reduction of reproduction was found at average exposure concentrations of 14 ng/l and higher. The onset of reproduction was clearly delayed in the highest group when compared to the control.

An increase of incidence of immobilised offspring was observed with increasing concentration.

Body length of adult daphnids was significantly reduced in the highest concentration.

4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) did not affect reproduction of *Daphnia magna* at 6.8 ng/l after 21 days of exposure (NOEC).

Exposure to average exposure concentrations of 14 ng/l induced significant inhibition of the reproductive capacity of the parental daphnids.

4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) caused not only reduction of reproduction but also delayed the onset of reproduction at the highest concentration tested.

Effect parameters (ng/l) obtained in this study are summarized in the table below

| Parameter                         | Concentration (ng/l) |
|-----------------------------------|----------------------|
| NOEC for reproduction             | 6.8                  |
| EC <sub>10</sub> for reproduction | 70 (1.3-150)         |
| EC <sub>50</sub> for reproduction | 209 (69-698)         |
| NOEC for mortality                | 550                  |
| NOEC for growth                   | 83                   |

() – 95% confidence intervals

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## 5. INTRODUCTION

### 5.1. Preface

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Study Monitor

Ms. P.A. Vernon

Test Facility

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The Netherlands

Study Director

M.A. Tobor-Kaplon, PhD.

Principal Scientist

K.A. Oudhoff, PhD.

Study Plan

Beginning: 29 October 2013  
Completion: 11 April 2014

### 5.2. Aim of the study

The purpose of the test was to evaluate the effects of the test substance on the mobility, the growth and the reproductive capacity of *Daphnia magna*. For this purpose, test organisms were exposed to aqueous solutions containing the test substance at various concentrations. The time of the first production of young, the number of young born, immobility and other signs of intoxication observed were compared with corresponding parameters in the controls.

### 5.3. Guidelines

The study procedures described in this report were based on the Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 211: "*Daphnia magna*, Reproduction Test", Adopted: October 2012.

In addition, the procedures were designed to meet the test methods prescribed by the following guidelines and guidance document:

- Commission Regulation (EC) No 440/2008 of 30 May 2008, Part C: Methods for the determination of ecotoxicity, Publication No. L142, C.20. "*Daphnia magna* Reproduction Test".
- ISO International Standard 10706: "Determination of long term toxicity of substances to *Daphnia magna* Straus (*Cladocera*, *Crustacea*)", 2000-03-30.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

### 5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the WIL Research Europe archives for a period of at least 2 years after finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. WIL Research Europe will retain information concerning decisions made.

Those specimens requiring refrigeration or freezing will be retained by WIL Research Europe for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

WIL Research Europe will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

### 5.5. Definitions

**Parent animals** are those female daphnids present at the start of the test and of which the reproductive output is the object of study.

**Offspring** are the young *Daphnia* produced by the parent animals in the course of the test.

The **No Observed Effect Concentration (NOEC)** is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect ( $p < 0.05$ ), within a stated exposure period.

**EC<sub>x</sub>** is the concentration of the test substance dissolved in water that results in a x per cent reduction in reproduction of *Daphnia magna* within a stated exposure period.

**Mortality.** An animal is recorded as dead when it is immobile, i.e. when it is not able to swim, or if there is no observed movement of appendages or postabdomen, within 15 seconds after gentle agitation of the test vessel.

### 5.6. Abbreviations:

**d** – nominal day of exposure

**n** – number of replicates (surviving daphnids)

**Std.Dev** – standard deviation

**CV** – coefficient of variation

**WSF** – Water Soluble Fraction

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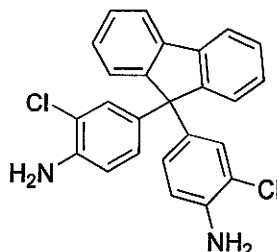
## 6. MATERIALS AND METHODS

### 6.1. Test Substance

#### 6.1.1. Test substance information

Identification  
Structure

4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline)



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|                                       |  |
|---------------------------------------|--|
| Molecular formula                     | C <sub>25</sub> H <sub>18</sub> Cl <sub>2</sub> N <sub>2</sub> |
| Molecular weight                      | 417.33   |
| CAS Number                            | 107934-68-9  |
| Description                           | White to off-white powder                                      |
| Batch                                 | DALA101444   |
| Purity/Composition                    | 97.3%  |
| Test substance storage                | At room temperature in the dark                                |
| Stable under storage conditions until | 31 August 2014 (expiry date)                                   |

#### 6.1.2. Study specific test substance information

|   |               |
|---|---------------|
| Purity/composition correction factor required | No            |
| Hygroscopic                                   | No            |
| Volatile                                      | No            |
| Stability in water                            | Not indicated |
| Solubility in water                           | No            |

### 6.2. Test system

|                      |  |
|----------------------|--|
| Species              | <i>Daphnia magna</i> (Crustacea, Cladocera) (Straus, 1820), at least third generation, obtained by acyclical parthenogenesis under specified breeding conditions.  |
| Source               | In-house laboratory culture with a known history.  |
| Reason for selection | This system has been selected as an internationally accepted invertebrate species.   |
| Validity of batch    | Daphnids originated from a healthy stock, 2 <sup>nd</sup> to 5 <sup>th</sup> brood, showing no signs of stress such as mortality >20%, presence of males, ephippia or discoloured animals and there was no delay in the production of the first brood. |
| Characteristics      | To initiate the test, young daphnids < 24 hours old were selected, from parental daphnids greater than two weeks old.  |

### 6.3. Breeding

|                     |   |
|---------------------|---|
| Start of each batch | With newborn daphnids, i.e. less than 3 days old, by placing them individually in 50 ml M7-medium |
|---------------------|---|

|                                       |   |
|---------------------------------------|---|
| Maximum age of the cultures           | 4 weeks   |
| Monitoring of the individual cultures | Three times a week the young are counted and the parental daphnids are transferred to new media.  |
| Temperature of medium                 | 18-22°C   |
| Feeding                               | Daily, a suspension of fresh water algae  |
| Validity of the cultures              | Historical data on the reproductive capacity are based on the numbers of living young counted three times a week in the individual cultures and tested to meet the validity criteria for survival and reproduction. |
| Medium                                | M7, as prescribed by Dr. Elendt-Schneider (Elendt, B.-P., 1990: Selenium deficiency in Crustacea. An ultrastructural approach to antennal damage in <i>Daphnia magna</i> Straus. Protoplasma 154, 25-33).           |

Composition of medium M7:

Adjusted ISO medium: the following chemicals (analytical grade) are dissolved in tap water purified by Reverse Osmosis (RO-water, GEON Waterbehandeling, Berkel-Enschot, The Netherlands):

|              |                                      |       |      |
|--------------|--------------------------------------|-------|------|
| Macro salts: | CaCl <sub>2</sub> ·2H <sub>2</sub> O | 211.5 | mg/l |
|              | MgSO <sub>4</sub> ·7H <sub>2</sub> O | 88.8  | mg/l |
|              | NaHCO <sub>3</sub>                   | 46.7  | mg/l |
|              | KCl                                  | 4.2   | mg/l |

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Medium M7: trace elements, macro nutrients and vitamins are added to freshly prepared ISO medium to reach the following concentrations:

|                  |   |        |      |
|------------------|---|--------|------|
| Trace elements:  | B   | 0.125  | mg/l |
|                  | Fe  | 0.05   | mg/l |
|                  | Mn  | 0.025  | mg/l |
|                  | Li, Rb and Sr                                       | 0.0125 | mg/l |
|                  | Mo  | 0.0063 | mg/l |
|                  | Br  | 0.0025 | mg/l |
|                  | Cu  | 0.0016 | mg/l |
|                  | Zn  | 0.0063 | mg/l |
|                  | Co and I  | 0.0025 | mg/l |
|                  | Se  | 0.0010 | mg/l |
|                  | V   | 0.0003 | mg/l |
|                  | Na <sub>2</sub> EDTA·2H <sub>2</sub> O              | 2.5    | mg/l |
| Macro nutrients: | Na <sub>2</sub> SiO <sub>3</sub> ·9H <sub>2</sub> O | 10.0   | mg/l |
|                  | NaNO <sub>3</sub>                                   | 0.27   | mg/l |
|                  | KH <sub>2</sub> PO <sub>4</sub>                     | 0.14   | mg/l |
|                  | K <sub>2</sub> HPO <sub>4</sub>                     | 0.18   | mg/l |
| Vitamins:        | Thiamine  | 75.0   | µg/l |
|                  | B <sub>12</sub>                                     | 1.0    | µg/l |
|                  | Biotin  | 0.75   | µg/l |

The hardness: 180 mg/l expressed as CaCO<sub>3</sub> and the pH: 7.7 ± 0.3.

#### 6.4. Preparation of test solutions

The standard test procedures required generation of test solutions, which should contain completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that disturb the test system should be prevented (e.g. precipitate or a film of

the test substance on the water surface). No correction was made for the purity/composition of the test substance.

The batch of 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) tested was a white to off-white powder with a purity of 97.3% and the substance was not completely soluble in test medium at the loading rates initially prepared.

Preparation started with a loading rate of 100 mg/l (range-finding test) or 5 mg/l (reproduction tests) applying a 30-minute treatment with ultrasonic waves followed by a 2-day period of magnetic stirring to ensure maximum dissolution in test medium. The obtained solutions containing undissolved material were filtered through a 0.45 µm membrane filter (Whatman, rc 55). Subsequently, the Water Soluble Fractions (WSFs) were used as the highest concentrations. Lower test concentrations were prepared by diluting the highest concentration in test medium. The final test solutions were all clear and colourless.

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#### 6.5. Range-finding test

No data were available from an acute toxicity test. Therefore, a preliminary test was performed prior to the reproduction test. The concentrations of 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) tested were: 0.10, 1.0, 10 and 100% of a WSF prepared at a loading rate of 100 mg/l. A control group was also included. Each concentration consisted of two replicates containing a total of ten daphnids (five each). The total test period was seven days. Test conditions were held as similar as possible to those applied in the reproduction test including feeding. Test solutions were renewed on days 2, 3 and 6 during the test. Samples for possible analysis were taken at days 0 (fresh), 2 (fresh and old), 3 (fresh and old) and 6 (old). Samples were taken from solutions containing the daphnia and food (algae), but also from an extra vessel incubated under the same conditions, but without daphnia and algae.

#### 6.6. First reproduction (limit) test

A first reproduction test was performed as a limit with a WSF prepared at a loading rate of 5.0 mg/l. The test was performed with procedures and conditions similar to those applied in the third reproduction test. This test was terminated on day 15 of exposure as a clear effect was observed at the limit concentration and, consequently, the NOEC for reproduction could not be determined.

#### 6.7. Second reproduction test

A second reproduction test was performed with procedures and conditions similar to those applied in the third reproduction test except for the concentration range. In this test daphnias were exposed to solutions containing 1.0, 3.2, 10, 32 and 100% of a WSF prepared at a loading rate of 5.0 mg/l. This test was terminated on day 18 of exposure as mortality in the control exceeded the allowed 20%.

#### 6.8. Third reproduction test

##### 6.8.1. Test concentrations

4,4'-(9H-fluoren-9-ylidene)  
bis(2-chloroaniline)

Solutions containing 0.16, 0.8, 4.0, 20 and 100% of a WSF prepared at a loading rate of 5.0 mg/l.

Controls

Test medium without test substance or other additives

##### 6.8.2. Test procedure and conditions

Test duration

21 days

Test type

Semi-static

Frequency of renewal

Every 48 hours

|                     |  |   |
|---------------------|--|---|
| Test vessels        | Volume: 60 ml (6 x Ø 3.5 cm), all-glass covered with a Perspex plate.  |   |
| Medium              | M7 <sup>1</sup>  |   |
| Experimental design | At the start of the experiment (nominal day 0) 10 neonate daphnids, less than one day old, per group were divided over ten vessels each containing a minimum of 50 ml test medium. The control group consisted of 20 daphnids.   |   |
| Light               | 16 h photoperiod daily;<br>intensity at the start: 616-669 lux<br>intensity at the end: 553-621lux   | PUBLIC COPY<br>DOES NOT CONTAIN<br>CONFIDENTIAL BUSINESS<br>INFORMATION |
| Feeding             | Twice daily an amount of 0.25 ml of <i>Chlorella pyrenoidosa</i> suspension <sup>2</sup> . On weekend days an amount of 0.50 ml was added in one single feed. This daily ration corresponded to 0.2 mg C/Daphnia/day, which is the recommended value for daily feeding per daphnid in the reproduction test according to the OECD Guideline 211. From day 14 onwards, the total daily amount was increased to 0.75 ml, based on expertise. This daily ration corresponded to 0.3 mg C/Daphnia/day. |   |

#### 6.8.3. Sampling for analysis of test concentrations

Samples for analysis were taken from all test concentrations and the control. In addition, the filter containing undissolved residue was kept for possible analysis. The method of analysis is described in the appended Analytical Report (APPENDIX 8).

|                     |   |
|---------------------|---|
| Sampling: Frequency | At the beginning and at the end of three intervals of 48 hours (nominal days 0 and 2, 4 and 6, 12 and 14, 18 and 20). |
| Volume              | 2.0 ml  |
| Storage             | Samples not analysed on the day of sampling were stored in a freezer until analysis.                                  |

At the end of the refreshment period, the replicates were pooled at each concentration before sampling.

Additionally, reserve samples of 2.0 ml were taken from all test solutions for possible analysis. If not already used, these samples were stored in a freezer for a maximum of three months after delivery of the draft report, pending on the decision of the sponsor for additional analysis.

#### 6.8.4. Measurements and recordings

##### Parental daphnids

|           |   |
|-----------|---|
| Condition | Every workday and upon renewal on non-workdays, the number of living, immobile and dead parental daphnids was recorded. Dead daphnids were removed when observed. |
|-----------|---|

|                                     |   |
|-------------------------------------|---|
| Presence of eggs in the brood pouch | Every workday and upon renewal on non-workdays. |
|-------------------------------------|---|

<sup>1</sup> Total (dissolved) organic carbon of the M7 medium used in the reproduction study was measured at the start of the test. Measurements were performed with a Shimadzu TOC-V<sub>COH</sub> total organic carbon analyzer combined with a Shimadzu ASI-V auto sampler (Shimadzu Kyoto, Japan). The sample volume was 40 ml and the number of repeats was at least 3.

<sup>2</sup> Algae suspensions were made by inoculating growth medium with *Chlorella pyrenoidosa* from a pure culture. The suspensions were continuously aerated and exposed to light (6000-10000 lux; 60-120 µE.m<sup>-2</sup>.s<sup>-1</sup>) in a climate room at a temperature of 23 ± 2°C for 2-3 weeks. After this period, the TOC concentration of the batch algal suspension was measured before use as feed in the reproduction study. The exact volume to be added per test vessel was then calculated.

Body length At the end of the test.

#### Offspring

Appearance first brood When observed.

Newborn daphnids Every workday and upon renewal on non-workdays, the number of newborn young was counted and the condition of the young recorded. Thereafter the young were removed.

Presence of unhatched eggs When observed.

Incidence of immobility When observed.

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#### Test medium

Temperature, oxygen and pH At the start of the test and just before and after each renewal in one of the vessels of each test group with surviving daphnids.

Hardness Once a week in fresh and old media from the control and the highest test concentration

Light At the start and the end of the test

### 6.9. Interpretation

#### 6.9.1. Data handling

The values for reproduction observed at various concentrations of the test substance were expressed as mean number of living young per parent. The mean values for reproduction at each concentration were compared to those recorded in the control on the various days of recording. Further, the length of the parental daphnids (day 21) exposed to the test substance were compared to the control.

#### Exposure concentrations

The exposure concentrations were calculated as

$$\frac{\sqrt{C_{t=0,\text{fresh}} \times C_{t=2,\text{old}}} + \sqrt{C_{t=4,\text{fresh}} \times C_{t=6,\text{old}}} + \sqrt{C_{t=12,\text{fresh}} \times C_{t=14,\text{old}}} + \sqrt{C_{t=18,\text{fresh}} \times C_{t=20,\text{old}}}}{4},$$

being the mathematical means of the concentrations of 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) measured in the samples taken from the freshly prepared solutions ( $C_{t=0}$ ,  $C_{t=4}$ ,  $C_{t=12}$  and  $C_{t=18}$ ) and from the 48-hour old solutions ( $C_{t=2}$ ,  $C_{t=6}$ ,  $C_{t=14}$  and  $C_{t=20}$ ).

In case concentrations measured were below the limit of quantification, the final exposure concentration(s) were taken as a factor of 2 below this limit. This procedure is based on the OECD "Guidance document on the use of the harmonised system for the classification of chemicals which are hazardous for the aquatic environment".

#### Statistical analysis

Following statistical procedures were used to determine the NOEC for reproduction and growth:

- Data distribution: Shapiro-Wilk's Test
- Homogeneity of variance: Levene's Test (with Residuals)
- Differences between treatments and the control: Williams Multiple Sequential t-test Procedure

Mortality of parental daphnids was analysed with Fisher's Exact Binomial Test with Bonferroni Correction.

EC-values for reproduction:

The EC<sub>10</sub> and EC<sub>50</sub> were determined using the Probit analysis using maximum likelihood regression with the probits of the percentages of cumulative reproduction at the end of the test as function of the logarithms of the corresponding concentrations.

All analyses were performed with ToxRat Professional 2.10.05 (ToxRat Solutions® GmbH, Germany).

**6.9.2. Electronic data capture**

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Observations/measurements in the study were recorded electronically using the following programme(s):

- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA): Temperature.
- Shimadzu TOC-Control V version 2.10 (Shimadzu, Kyoto, Japan): carbon analysis.

**6.9.3. Acceptability of the test**

1. The mortality of the parent animals (female *Daphnia*) in the control did not exceed 20% at the end of the test (5%).
2. The average cumulative number of young per female in the controls after 21 days was  $\geq 60$  (84).

**6.10. List of deviations****6.10.1. List of protocol deviations**

1. The solutions were renewed on days 2, 3 and 6 instead of 1, 3 and 6 in the range-finding test. The sampling points changed accordingly.  
Evaluation: This had no impact on the results.
2. Third reproduction test: on day 14 of exposure daphnids were fed only once and therefore, received 0.1 instead of 0.3 mg C per day.  
Evaluation: *Daphnia* are fed at libitum and received the required amount of food next day.

The study integrity was not adversely affected by the deviations.

**6.10.2. List of standard operating procedures deviations**

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

**7. RESULTS****7.1. Range-finding test**

Table 1 summarizes the daily observations of mortality and reproductive potency of surviving parental daphnids during the 7-day range-finding test. No mortality was observed at any concentration tested during the entire exposure period. No reproduction was observed in the range-finding test. There were no clear differences in the appearance of eggs in the brood pouch between different concentrations.

The analytical results are summarized in Table 2 of the appended Analytical Report.

The actual concentrations measured in the undiluted WSF at the start of each refreshment period were at the level of 1.0 to 2.0 µg/l. The measured concentrations were at the level of 85-110% of initial at the end of refreshment periods except for the 1 day refreshment period where no concentration could be determined at the end of the renewal. The concentrations in abiotic controls were less stable, i.e. decreased to 54 to 80% of initial.

Test conditions during the range-finding test were maintained within the limits prescribed by the protocol.

**Table 1 Mortality and reproductive potency during the range-finding test**

| Test substance <sup>1</sup><br>WSF prepared at<br>100 mg/l | Number<br>Daphnia<br>exposed | Cumulative number of dead/immobile parental daphnids |       |       |       |       |       |       |
|--|------------------------------|--|-------|-------|-------|-------|-------|-------|
|  |                              | Day 1  | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Control  | 5                            | 0  | 0     | 0     | 0     | 0     | 0     | 0 (4) |
|  | 5                            | 0  | 0     | 0     | 0     | 0 (1) | 0 (1) | 0 (5) |
|  | 5                            | 0  | 0     | 0     | 0     | 0 (1) | 0 (2) | 0 (5) |
| 0.10   | 5                            | 0  | 0     | 0     | 0     | 0     | 0 (2) | 0 (4) |
|  | 5                            | 0  | 0     | 0     | 0     | 0     | 0     | 0 (5) |
| 1.0  | 5                            | 0  | 0     | 0     | 0     | 0     | 0 (1) | 0 (4) |
|  | 5                            | 0  | 0     | 0     | 0     | 0     | 0 (2) | 0 (5) |
| 10   | 5                            | 0  | 0     | 0     | 0     | 0     | 0 (1) | 0 (5) |
|  | 5                            | 0  | 0     | 0     | 0     | 0     | 0 (2) | 0 (5) |
| 100  | 5                            | 0  | 0     | 0     | 0     | 0     | 0     | 0 (5) |

1. 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline)

( ) number of parents with eggs in their brood pouch

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**7.2. First reproduction (limit) test**

The first reproduction test was performed as a limit with the limit concentration being a WSF prepared at a loading rate of 5.0 mg/l. The loading rate was chosen in consultation with the sponsor.

**7.2.1. Mortality of parental daphnids**

Table 2 summarizes the survival of parental daphnids during the exposure. No mortality was observed in the control during 14 days of exposure. In the highest concentration three daphnids died during the exposure.

**Table 2 Survival of parental daphnids in the first (limit) reproduction test**

| Nominal day | Test substance <sup>1,2</sup> |     |
|-------------|-------------------------------|-----|
|             | Control                       | 100 |
| 0           | 20                            | 20  |
| 4           | 20                            | 19  |
| 6           | 20                            | 18  |
| 7           | 20                            | 17  |
| 8           | 20                            | 17  |
| 10          | 20                            | 17  |
| 11          | 20                            | 17  |
| 12          | 20                            | 17  |
| 13          | 20                            | 17  |
| 14          | 20                            | 17  |

1. 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline)

2. % WAF prepared at a loading rate of 5.0 mg/l

**7.2.2. Reproduction**

Group mean cumulative numbers of offspring per parental daphnids at day 14 of exposure are shown in Table 3. The individual data recorded per parent are kept in the study files and are not reported.

**Table 3** Group mean cumulative number of juveniles per surviving parent and reduction of reproduction at day 14 in the first (limit) reproduction test

|              | Test substance <sup>1,2</sup> |       |
|--------------|-------------------------------|-------|
|              | Control                       | 100   |
| Mean:        | 35.1                          | 0.6   |
| Std.Dev.:    | 9.1                           | 1.8   |
| n:           | 20                            | 17    |
| CV:          | 25.9                          | 283.6 |
| % Reduction: | n.a.                          | 98    |

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1. 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline)  
2. % WAF prepared at a loading rate of 5.0 mg/l

Because a clear effect in reproduction was observed after 14 days of exposure the test was terminated and repeated with a range of concentrations.

An increase in incidence of immobilised offspring was observed in the limit concentration. Table 4 summarizes the observations made in this test.

**Table 4** Mean cumulative number of immobilised offspring per parental daphnid and aborted eggs in the first (limit) reproduction test

| Abnormality  | Test substance <sup>1,2</sup> |      |
|--------------|-------------------------------|------|
|              | Control                       | 100  |
| Immobile     | 0.80                          | 20   |
| Aborted eggs | n.o.                          | n.o. |

1. 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline)  
2. % WAF prepared at a loading rate of 5.0 mg/l  
n.o. – not observed

### 7.3. Second reproduction test

#### 7.3.1. Mortality of parental daphnids

Table 5 summarizes the survival of parental daphnids during the exposure. Until day 16 mortality in the control was limited to one daphnid. Moreover one daphnid died in the lowest concentration and two in the highest concentration. At day 18 of exposure four additional daphnids were found dead in the control and the test was therefore terminated.

**Table 5** Survival of parental daphnids in the second reproduction test

| Nominal day | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) <sup>1</sup> |      |      |      |      |      |
|-------------|--|------|------|------|------|------|
|             | Control  | 1.0  | 3.2  | 10   | 32   | 100  |
| 0           | 20   | 10   | 10   | 10   | 10   | 10   |
| 6           | 20   | 10   | 10   | 10   | 10   | 9    |
| 8           | 20   | 10   | 10   | 10   | 10   | 9    |
| 10          | 20   | 9    | 10   | 10   | 10   | 8    |
| 11          | 20   | 9    | 10   | 10   | 10   | 8    |
| 12          | 19   | 9    | 10   | 10   | 10   | 8    |
| 13          | 19   | 9    | 10   | 10   | 10   | 8    |
| 14          | 19   | 9    | 10   | 10   | 10   | 8    |
| 15          | 19   | 9    | 10   | 10   | 10   | 8    |
| 16          | 19   | 9    | 10   | 10   | 10   | 8    |
| 18          | 15   | n.d. | n.d. | n.d. | n.d. | n.d. |

1. % WAF prepared at a loading rate of 5.0 mg/l  
n.d. not determined; after mortality in the control treatment was assessed the remaining concentrations were not checked for mortality.

### 7.3.2. Reproduction

Group mean cumulative numbers of offspring per parental daphnids at the end of the test are shown in Table 6. The individual data recorded per parent are kept in the study files and are not reported.

Statistical analysis of the cumulative reproduction at day 16 showed that the reproduction of the daphnids was significantly reduced already at the lowest concentration. Moreover, a very shallow dose-response was observed at the four lowest concentrations tested. Therefore, it was decided, after consultation with the sponsor, that the third reproduction test would be performed with a spacing factor larger than 3.2, namely 5. This should ensure determination of a NOEC for reproduction.

**Table 6** Group mean cumulative number of juveniles per surviving parent and reduction of reproduction at day 16 in the second reproduction test

|              | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) <sup>1</sup> |     |     |     |     |     |
|--------------|--|-----|-----|-----|-----|-----|
|              | Control  | 1.0 | 3.2 | 10  | 32  | 100 |
| Mean:        | 56   | 48  | 48  | 46  | 42  | 2.0 |
| Std.Dev.:    | 9.0  | 7.8 | 3.7 | 4.0 | 11  | 4.0 |
| n:           | 19   | 9   | 10  | 10  | 10  | 8.0 |
| CV:          | 16   | 16  | 7.8 | 8.7 | 26  | 228 |
| % Reduction: | -  | 15* | 16* | 19* | 26* | 96* |

1. % WAF prepared at a loading rate of 5.0 mg/l

\* effect is statistically significant

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During this test a clear-dose depended incidence of immobilised offspring was observed. Table 7 summarizes the observations made in this test.

**Table 7** Mean cumulative number of immobilised offspring per parental daphnid and aborted eggs in the second reproduction test

| Abnormality  | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) <sup>1</sup> |      |      |      |      |     |
|--------------|--|------|------|------|------|-----|
|              | Control  | 1.0  | 3.2  | 10   | 32   | 100 |
| Immobile     | 0.16   | 1.1  | 0.4  | 5.9  | 9.5  | 27  |
| Aborted eggs | n.o.   | n.o. | n.o. | n.o. | n.o. | 8   |

1. % WAF prepared at a loading rate of 5.0 mg/l

n.o. – not observed

A delay in the age at the first reproduction was observed in this test. The majority of the daphnids in the control and the four lowest concentrations started to reproduce around day 11 of exposure, whereas in the highest concentration the appearance of viable offspring occurred on around day 16 of exposure.

### 7.4. Third reproduction test

#### 7.4.1. Measured concentrations

The results of analysis of the samples taken during the third reproduction test are described in Table 4 of the appended Analytical Report. Note, that in this test a more sensitive analytical method was employed than in the range-finding test.

The concentrations measured in freshly prepared WSFs at the beginning of each renewal period ranged between 926 and 1697 ng/l (average  $1268 \pm 328$  (StDev) ng/l). Measured concentrations in the undiluted WSF were at a level of 10-39% of initial at the end of each refreshment period. The actual concentrations in the remaining groups showed a similar variability. Therefore, average exposure concentrations were calculated using the formula from paragraph 6.9.1 (see Table 8).

**Table 8** Nominal and mean measured exposure concentrations in the third reproduction test

| Test substance <sup>1</sup><br>% WSF prep. at<br>5.0 mg/l | Mean exposure concentration (ng/l) |                  |                  |           | Average exposure<br>concentration<br>(ng/l) |
|---|------------------------------------|------------------|------------------|-----------|---|
|   | Day 0-2                            | Day 6-8          | Day 12-14        | Day 18-20 |   |
| 0.16  | 7.6                                | 5.0 <sup>2</sup> | 5.0 <sup>2</sup> | 7.1       | 6.2   |
| 0.80  | 5.0 <sup>2</sup>                   | 9.8              | 5.0 <sup>2</sup> | 7.3       | 6.8   |
| 4.0   | 13                                 | 12               | 14               | 15        | 14  |
| 20  | 122                                | 82               | 62               | 64        | 83  |
| 100   | 459                                | 615              | 706              | 418       | 550   |

1. % WAF prepared at a loading rate of 5.0 mg/l

2. Half of LOQ (LOQ=10 ng/l)

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One parental daphnid died during the test period in the control treatment (see Table 9). Hence, parental mortality did not exceed 20% in the control. Three parental daphnids died in each, 14 and 550 ng/l whereas all daphnia survived the exposure in the remaining groups. This mortality was not statistically different from the control.

**Table 9** Survival of parental daphnids in the third reproduction test

| Nominal day | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), Concentration (ng/l) |     |     |    |    |     |
|-------------|---|-----|-----|----|----|-----|
|             | Control   | 6.2 | 6.8 | 14 | 83 | 550 |
| 0           | 20  | 10  | 10  | 10 | 10 | 10  |
| 5           | 20  | 10  | 10  | 10 | 10 | 9   |
| 8           | 19  | 10  | 10  | 10 | 10 | 9   |
| 10          | 19  | 10  | 10  | 10 | 10 | 9   |
| 11          | 19  | 10  | 10  | 10 | 10 | 9   |
| 12          | 19  | 10  | 10  | 10 | 10 | 8   |
| 13          | 19  | 10  | 10  | 10 | 10 | 8   |
| 14          | 19  | 10  | 10  | 10 | 10 | 8   |
| 16          | 19  | 10  | 10  | 8  | 10 | 7   |
| 17          | 19  | 10  | 10  | 8  | 10 | 7   |
| 18          | 19  | 10  | 10  | 8  | 10 | 7   |
| 19          | 19  | 10  | 10  | 7  | 10 | 7   |
| 20          | 19  | 10  | 10  | 7  | 10 | 7   |
| 21          | 19  | 10  | 10  | 7  | 10 | 7   |

**7.4.3. Time to first reproduction**

On average, the first reproduction took place around day 10 of exposure in the control and all concentrations besides the highest one. The appearance of the first, viable, brood in the highest concentration took place on day 19 of exposure. Therefore, it can be concluded that the test substance causes a delay in reproduction of *Daphnia magna*.

**7.4.4. Reproduction**

Group mean cumulative numbers of offspring per parental daphnids at the end of the test are shown in Table 10 and Figure 1. The data recorded per parent are presented in Table 14, APPENDIX 1.

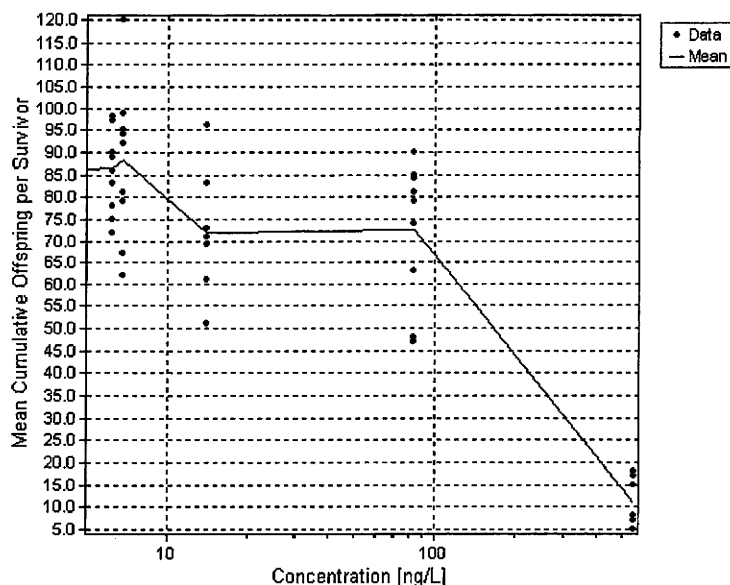
On average, 84 offspring were produced per surviving daphnid in the control treatment. Similar reproduction was observed at the two lowest concentrations. In the three highest concentrations significant reduction of reproduction was observed (see APPENDIX 3 for statistical analysis).

The dose-response was very shallow, which was similar to the observation made in the previous test.

**Table 10** Group mean cumulative number of juveniles per surviving parent and reduction of reproduction at the end of the third reproduction test

|              | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |      |      |     |     |     |
|--------------|---|------|------|-----|-----|-----|
|              | Control   | 6.2  | 6.8  | 14  | 83  | 550 |
| Mean:        | 84  | 87   | 88   | 72  | 73  | 11  |
| Std.Dev.:    | 13  | 10   | 17   | 15  | 15  | 6   |
| n:           | 19  | 10   | 10   | 7   | 10  | 7   |
| CV:          | 16  | 11   | 19   | 20  | 21  | 54  |
| % Reduction: | n.a.  | -3.0 | -4.8 | 14* | 14* | 87* |

n.a. — not applicable

\* Statistically significant ( $p < 0.05$ )

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**Figure 1** Cumulative mean number of living young per parent at different test groups of 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) (in mg/l) during the 21-day test period (third reproduction test).

Numbers of immobilised offspring or aborted eggs observed during the study are summarized in Table 11. Distribution over replicates and time is given in Table 14.

A dose-related increase in appearance of immobile offspring was observed in this study, confirming earlier observations. Note that most immobile offspring (in the control 98%) in all groups except for the highest concentration were born during the last two days of exposure. In the highest concentration immobile offspring were born already at day 10 of exposure. No explanation can be given for the high number of immobile offspring in the control on the last day of the exposure, however, this observation does not invalidate the study.

**Table 11** Mean cumulative number of immobilised offspring per parental daphnid and aborted eggs in the third reproduction test

|              | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |     |     |      |      |      |
|--------------|---|-----|-----|------|------|------|
| Abnormality  | Control   | 6.2 | 6.8 | 14   | 83   | 550  |
| Immobile     | 6.0   | 7.7 | 3.0 | 22   | 25   | 43   |
| Aborted eggs | n.o.  | 2   | 2   | n.o. | n.o. | n.o. |

n.o. — not observed

#### 7.4.5. Body length

The group mean body lengths of the surviving daphnids per concentration measured at the end of the test and the relative reduction of body lengths compared to the control are summarised in Table 12 (see APPENDIX 5 for the individual body lengths of the surviving parental daphnids).

Statistically significant reduction of growth was found at the highest concentration.

**Table 12** Group mean body lengths and reduction of growth of parental daphnids at the end of the third reproduction test.

| Test substance <sup>1</sup><br>concentration<br>(ng/l) | Mean | Std. Dev. | n  | %Reduction |
|--|------|-----------|----|------------|
| Control  | 4.18 | 0.167     | 19 | 0.0        |
| 6.2  | 4.16 | 0.126     | 10 | 0.4        |
| 6.8  | 4.10 | 0.177     | 10 | 1.8        |
| 14   | 4.15 | 0.145     | 7  | 0.6        |
| 83   | 4.26 | 0.132     | 10 | -1.9       |
| 550  | 3.69 | 0.140     | 7  | 11.8*      |

1. 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline)

\* Statistically significant ( $p < 0.05$ )

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#### 7.4.6. Determination of effect concentrations

Table 13 shows the effect parameters based on measured concentrations, see also APPENDIX 2, APPENDIX 3 and APPENDIX 6.

**Table 13** Effect parameters

| Parameter                         | Concentration (ng/l) |
|-----------------------------------|----------------------|
| NOEC for reproduction             | 6.8                  |
| EC <sub>10</sub> for reproduction | 70 (1.3-150)         |
| EC <sub>50</sub> for reproduction | 209 (69-698)         |
| NOEC for mortality                | 550                  |
| NOEC for growth                   | 83                   |

() – 95% confidence intervals

#### 7.4.7. Experimental conditions

The pH values recorded during the test are presented in APPENDIX 7, Table 18. The pH remained within the range of 7.8-9.0 throughout the test and thus was maintained within the limits prescribed by the protocol (6.0-9.0, constant within 1.5 units).

The dissolved oxygen concentrations measured during the study are presented in APPENDIX 7, Table 19. The oxygen concentration in all test solutions remained within the range of 8.7-11 mg/l during the exposure period and thus complied to the requirements as laid down in the protocol ( $> 3$  mg/l).

Temperatures recorded in the test media are shown in APPENDIX 7, Table 20. The temperatures in the test media varied between 19-21°C. The temperature continuously measured in a temperature control vessel varied between 20-21°C during the test, and complied with the requirements as laid down in the protocol (18-22°C, constant within 2°C).

The results of the measurements of total hardness are presented in APPENDIX 7, Table 21. Total hardness varied between 179-214 mg calcium carbonate per litre, and thus complied to the requirements as laid down in the protocol ( $> 140$  mg CaCO<sub>3</sub> per liter).

The total dissolved organic carbon content of the M7 medium was 0.12 mg/l. This complied with the requirements as laid down in the guidelines (TOC < 2 mg/l).

## 8. CONCLUSION

Under the conditions of the present study 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) did not affect reproduction of *Daphnia magna* at 6.8 ng/l after 21 days of exposure (NOEC).

Exposure to average exposure concentrations of 14 ng/l and higher induced significant inhibition of the reproductive capacity of the parental daphnids.

4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline) caused not only reduction of reproduction but also delayed the onset of reproduction at the highest concentration tested.

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## APPENDIX 1 NUMBER OF LIVING NEWBORN DAPHNIDS PER PARENT

Table 14 Number of living newborn per parent per day

| Nominal day | Replicate | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |        |        |        |        |        |
|-------------|-----------|---|--------|--------|--------|--------|--------|
|             |           | Control   | 6.2    | 6.8    | 14     | 83     | 550    |
| 0           | 1         | 0   | 0      | 0      | 0      | 0      | 0      |
|             | 2         | 0   | 0      | 0      | 0      | 0      | 0      |
|             | 3         | 0   | 0      | 0      | 0      | 0      | 0      |
|             | 4         | 0   | 0      | 0      | 0      | 0      | 0      |
|             | 5         | 0   | 0      | 0      | 0      | 0      | 0      |
|             | 6         | 0   | 0      | 0      | 0      | 0      | 0      |
|             | 7         | 0   | 0      | 0      | 0      | 0      | 0      |
|             | 8         | 0   | 0      | 0      | 0      | 0      | 0      |
|             | 9         | 0   | 0      | 0      | 0      | 0      | 0      |
|             | 10        | 0   | 0      | 0      | 0      | 0      | 0      |
|             | 11        | 0   |        |        |        |        |        |
|             | 12        | 0   |        |        |        |        |        |
|             | 13        | 0   |        |        |        |        |        |
|             | 14        | 0   |        |        |        |        |        |
|             | 15        | 0   |        |        |        |        |        |
|             | 16        | 0   |        |        |        |        |        |
|             | 17        | 0   |        |        |        |        |        |
|             | 18        | 0   |        |        |        |        |        |
|             | 19        | 0   |        |        |        |        |        |
|             | 20        | 0   |        |        |        |        |        |
| 10          | 1         | 0   | 5      | 0      | 0      | 0      | 0      |
|             | 2         | 0   | 21     | 18     | 1      | 0, 2i  | 0, 5i  |
|             | 3         | 0   | 19, 1i | 14     | 0      | 0, 2i  | 0, 11i |
|             | 4         | 15  | 0      | 14     | 0      | 0, 1i  | 0, 9i  |
|             | 5         | 0   | 15, 2i | 15, 1i | 1      | 15, 1i | 0, 18i |
|             | 6         | 19  | 19     | 0      | 19, 1i | 1, 1i  | 0, 2i  |
|             | 7         | 8   | 18     | 0      | 18, 3i | 22     | 0, 11i |
|             | 8         | 2, 1i   | 0      | 15, 1i | 0      | 18     | 0, 10i |
|             | 9         | 20  | 2      | 21     | 0      | 0      | 0, 5i  |
|             | 10        | 20  | 19, 1i | 21     | 0      | 24     | 2      |
|             | 11        | 0   |        |        |        |        |        |
|             | 12        | 0   |        |        |        |        |        |
|             | 13        | 0   |        |        |        |        |        |
|             | 14        | 16  |        |        |        |        |        |
|             | 15        | -1  |        |        |        |        |        |
|             | 16        | 16  |        |        |        |        |        |
|             | 17        | 14  |        |        |        |        |        |
|             | 18        | 6   |        |        |        |        |        |
|             | 19        | 0   |        |        |        |        |        |
|             | 20        | 0   |        |        |        |        |        |

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1. Daphnia died on nominal day 8

2. Daphnia died on nominal day 5

+/- : dead parental daphnid

E: number of aborted eggs

i: number of immobile offspring

| Nominal day | Replicate | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |     |        |        |    |        |
|-------------|-----------|---|-----|--------|--------|----|--------|
|             |           | Control   | 6.2 | 6.8    | 14     | 83 | 550    |
| 11          | 1         | 21  | 0   | 19     | 22     | 23 | 0      |
|             | 2         | 20  | 0   | 0      | 16     | 22 | 0      |
|             | 3         | 17  | 0   | 0      | 19     | 19 | 0      |
|             | 4         | 0   | 21  | 0      | 13, 1E | 22 | 0      |
|             | 5         | 22  | 0   | 0      | 22     | 0  | 0      |
|             | 6         | 0   | 0   | 13     | 0      | 18 | 0      |
|             | 7         | 0   | 0   | 13     | 0      | 0  | 0      |
|             | 8         | 0   | 20  | 0      | 12, 1E | 0  | 0      |
|             | 9         | 0   | 16  | 0      | 19     | 17 | 0      |
|             | 10        | 0   | 0   | 0      | 19     | 0  | -      |
|             | 11        | 0   |     |        |        |    |        |
|             | 12        | 15  |     |        |        |    |        |
|             | 13        | 18  |     |        |        |    |        |
|             | 14        | 0   |     |        |        |    |        |
|             | 15        | -   |     |        |        |    |        |
|             | 16        | 0   |     |        |        |    |        |
|             | 17        | 0   |     |        |        |    |        |
|             | 18        | 0   |     |        |        |    |        |
|             | 19        | 16  |     |        |        |    |        |
|             | 20        | 18  |     |        |        |    |        |
| 12          | 1         | 0   | 22  | 0      | 1      | 1  | +      |
|             | 2         | 0   | 0   | 0      | 0      | 0  | 0      |
|             | 3         | 0   | 18  | 24     | 0      | 0  | 0      |
|             | 4         | 26  | 1   | 20     | 0      | 1  | 0      |
|             | 5         | 0   | 21  | 15, 1E | 0      | 20 | 0      |
|             | 6         | 0   | 0   | 0      | 19, 1i | 1  | 0, 12i |
|             | 7         | 0   | 0   | 0      | 27     | 1  | 0      |
|             | 8         | 19  | 0   | 20, 1i | 1      | 0  | 0      |
|             | 9         | 21  | 0   | 0      | 0      | 0  | 0, 2i  |
|             | 10        | 0   | 18  | 28     | 1      | 0  | -      |
|             | 11        | 18  |     |        |        |    |        |
|             | 12        | 1   |     |        |        |    |        |
|             | 13        | 0   |     |        |        |    |        |
|             | 14        | 15  |     |        |        |    |        |
|             | 15        | -   |     |        |        |    |        |
|             | 16        | 0   |     |        |        |    |        |
|             | 17        | 20  |     |        |        |    |        |
|             | 18        | 0   |     |        |        |    |        |
|             | 19        | 0   |     |        |        |    |        |
|             | 20        | 0   |     |        |        |    |        |

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+/- : dead parental daphnid  
E: number of aborted eggs  
i: number of immobile offspring

| Nominal day | Replicate | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |        |     |        |    |        |
|-------------|-----------|---|--------|-----|--------|----|--------|
|             |           | Control   | 6.2    | 6.8 | 14     | 83 | 550    |
| 13          | 1         | 0   | 0      | 0   | 0      | 0  | -      |
|             | 2         | 0   | 23     | 29  | 0      | 0  | 0, 6i  |
|             | 3         | 0   | 0      | 0   | 0      | 0  | 0, 7i  |
|             | 4         | 0   | 0      | 0   | 0      | 0  | 0, 9i  |
|             | 5         | 0   | 0      | 0   | 0      | 0  | 0, 10i |
|             | 6         | 29  | 22     | 0   | 0      | 0  | 0      |
|             | 7         | 20  | 21     | 0   | 0      | 29 | 0, 9i  |
|             | 8         | 0   | 0      | 0   | 0      | 22 | 0, 9i  |
|             | 9         | 0   | 0      | 25  | 0      | 0  | 0, 8i  |
|             | 10        | 23  | 0      | 3   | 0      | 22 | -      |
|             | 11        | 0   |        |     |        |    |        |
|             | 12        | 0   |        |     |        |    |        |
|             | 13        | 0   |        |     |        |    |        |
|             | 14        | 0   |        |     |        |    |        |
|             | 15        | -   |        |     |        |    |        |
|             | 16        | 21  |        |     |        |    |        |
|             | 17        | 0   |        |     |        |    |        |
|             | 18        | 18  |        |     |        |    |        |
|             | 19        | 0   |        |     |        |    |        |
|             | 20        | 0   |        |     |        |    |        |
| 14          | 1         | 20  | 0      | 16  | 21     | 27 | -      |
|             | 2         | 18  | 0      | 0   | 19     | 19 | 0      |
|             | 3         | 21  | 0      | 0   | 20     | 22 | 0      |
|             | 4         | 0   | 25, 1E | 0   | 19     | 20 | 0, 4i  |
|             | 5         | 25  | 0      | 0   | 18     | 0  | 0, 3i  |
|             | 6         | 0   | 0      | 18  | 0      | 20 | 0      |
|             | 7         | 0   | 0      | 12  | 0      | 0  | 0, 2i  |
|             | 8         | 0   | 20     | 0   | 19     | 0  | 0, 4i  |
|             | 9         | 0   | 28     | 0   | 26     | 29 | 0, 1i  |
|             | 10        | 0   | 0      | 0   | 26, 1i | 0  | -      |
|             | 11        | 0   |        |     |        |    |        |
|             | 12        | 1   |        |     |        |    |        |
|             | 13        | 23  |        |     |        |    |        |
|             | 14        | 0   |        |     |        |    |        |
|             | 15        | -   |        |     |        |    |        |
|             | 16        | 0   |        |     |        |    |        |
|             | 17        | 0   |        |     |        |    |        |
|             | 18        | 0   |        |     |        |    |        |
|             | 19        | 17  |        |     |        |    |        |
|             | 20        | 20  |        |     |        |    |        |

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+/- : dead parental daphnid  
E: number of aborted eggs  
i: number of immobile offspring

| Nominal day | Replicate | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |        |     |        |         |        |
|-------------|-----------|---|--------|-----|--------|---------|--------|
|             |           | Control   | 6.2    | 6.8 | 14     | 83      | 550    |
| 16          | 1         | 0   | 24     | 0   | +      | 0       | -      |
|             | 2         | 0   | 26     | 25  | 0      | 0       | 0, 12i |
|             | 3         | 1   | 20, 1i | 28  | 0      | 0       | +      |
|             | 4         | 22  | 0      | 25  | 0      | 0       | 0, 7i  |
|             | 5         | 0   | 26     | 34  | 0      | 30      | 0, 16i |
|             | 6         | 30, 1i  | 26     | 0   | 35     | 0       | 0, 16i |
|             | 7         | 28  | 31     | 0   | 24     | 26, 6i  | 0, 15i |
|             | 8         | 27  | 0      | 25  | 0      | 22      | 0, 13i |
|             | 9         | 29  | 0      | 30  | 1      | 0       | 0, 5i  |
|             | 10        | 26  | 28     | 34  | +      | 26      | -      |
|             | 11        | 21  |        |     |        |         |        |
|             | 12        | 21  |        |     |        |         |        |
|             | 13        | 0   |        |     |        |         |        |
|             | 14        | 18  |        |     |        |         |        |
|             | 15        | -   |        |     |        |         |        |
|             | 16        | 16  |        |     |        |         |        |
|             | 17        | 26  |        |     |        |         |        |
|             | 18        | 22  |        |     |        |         |        |
|             | 19        | 0   |        |     |        |         |        |
|             | 20        | 0   |        |     |        |         |        |
| 17          | 1         | 28  | 0      | 0   | -      | 28      | -      |
|             | 2         | 24  | 0      | 0   | 7, 31i | 33      | 0      |
|             | 3         | 24  | 0      | 0   | 25, 8i | 2, 26i  | -      |
|             | 4         | 0   | 4      | 0   | 25     | 15, 27i | 0      |
|             | 5         | 39  | 0      | 0   | 28     | 0       | 0      |
|             | 6         | 0   | 0      | 29  | 0      | 5       | 0      |
|             | 7         | 0   | 0      | 21  | 0      | 0       | 0      |
|             | 8         | 0   | 31     | 0   | 40     | 0       | 0      |
|             | 9         | 0   | 2      | 0   | 37     | 28, 4i  | 0      |
|             | 10        | 0   | 0      | 0   | -      | 0       | -      |
|             | 11        | 0   |        |     |        |         |        |
|             | 12        | 0   |        |     |        |         |        |
|             | 13        | 13  |        |     |        |         |        |
|             | 14        | 0   |        |     |        |         |        |
|             | 15        | -   |        |     |        |         |        |
|             | 16        | 0   |        |     |        |         |        |
|             | 17        | 0   |        |     |        |         |        |
|             | 18        | 0   |        |     |        |         |        |
|             | 19        | 39  |        |     |        |         |        |
|             | 20        | 0   |        |     |        |         |        |

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+/- : dead parental daphnid  
E: number of aborted eggs  
i: number of immobile offspring

| Nominal day | Replicate | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |         |        |    |        |        |
|-------------|-----------|---|---------|--------|----|--------|--------|
|             |           | Control   | 6.2     | 6.8    | 14 | 83     | 550    |
| 18          | 1         | 0   | 21      | 30     | -  | 4      | -      |
|             | 2         | 0   | 0       | 0      | 0  | 0      | 0      |
|             | 3         | 3   | 0       | 0      | 0  | 0      | -      |
|             | 4         | 17  | 24      | 18     | 0  | 0      | 0      |
|             | 5         | 0   | 16      | 31     | 0  | 0      | 0      |
|             | 6         | 0   | 0       | 0      | 23 | 3, 27i | 0, 23i |
|             | 7         | 0   | 0       | 0      | 7  | 0      | 0      |
|             | 8         | 27  | 0       | 32     | 0  | 0      | 0      |
|             | 9         | 0   | 29      | 0      | 0  | 0      | 0      |
|             | 10        | 0   | 0       | 0      | -  | 0      | -      |
|             | 11        | 18  |         |        |    |        |        |
|             | 12        | 27  |         |        |    |        |        |
|             | 13        | 26  |         |        |    |        |        |
|             | 14        | 28  |         |        |    |        |        |
|             | 15        | -   |         |        |    |        |        |
|             | 16        | 0   |         |        |    |        |        |
|             | 17        | 36  |         |        |    |        |        |
|             | 18        | 0   |         |        |    |        |        |
|             | 19        | 0   |         |        |    |        |        |
|             | 20        | 36  |         |        |    |        |        |
| 19          | 1         | 0   | 0       | 0      | -  | 0      | -      |
|             | 2         | 0   | 28      | 22     | 0  | 0      | 7, 12i |
|             | 3         | 0   | 29      | 26, 1i | 0  | 0      | -      |
|             | 4         | 0   | 0       | 2      | 0  | 0      | 8, 7i  |
|             | 5         | 0   | 12      | 0      | 0  | 20, 5i | 5, 8i  |
|             | 6         | 4   | 23      | 0      | 0  | 0      | 0      |
|             | 7         | 27  | 13, 15i | 0      | +  | 3, 28i | 5, 7i  |
|             | 8         | 0   | 0       | 0      | 0  | 17, 8i | 15     |
|             | 9         | 32  | 0       | 0      | 0  | 0      | 18     |
|             | 10        | 30  | 32      | 34, 3i | -  | 18, 8i | -      |
|             | 11        | 0   |         |        |    |        |        |
|             | 12        | 0   |         |        |    |        |        |
|             | 13        | 0   |         |        |    |        |        |
|             | 14        | 0   |         |        |    |        |        |
|             | 15        | -   |         |        |    |        |        |
|             | 16        | 33  |         |        |    |        |        |
|             | 17        | 0   |         |        |    |        |        |
|             | 18        | 22  |         |        |    |        |        |
|             | 19        | 0   |         |        |    |        |        |
|             | 20        | 0   |         |        |    |        |        |

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+/- : dead parental daphnid  
E: number of aborted eggs  
i: number of immobile offspring

| Nominal day | Replicate | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |        |        |        |        |        |
|-------------|-----------|---|--------|--------|--------|--------|--------|
|             |           | Control   | 6.2    | 6.8    | 14     | 83     | 550    |
| 20          | 1         | 0   | 0      | 0      | -      | 0      | -      |
|             | 2         | 0   | 0      | 0      | 8, 1i  | 0      | 0      |
|             | 3         | 0   | 0      | 0      | 0      | 4, 10i | -      |
|             | 4         | 0   | 0      | 0      | 4, 19i | 5, 11i | 0      |
|             | 5         | 25  | 0      | 0      | 2, 23i | 0      | 0      |
|             | 6         | 22  | 8      | 21     | 0      | 0      | 0      |
|             | 7         | 0   | 0      | 16     | -      | 0      | 0      |
|             | 8         | 0   | 18     | 0      | 1, 22i | 0      | 0      |
|             | 9         | 0   | 0      | 23     | 0      | 0      | 0      |
|             | 10        | 0   | 0      | 0      | -      | 0      | -      |
|             | 11        | 0   |        |        |        |        |        |
|             | 12        | 0   |        |        |        |        |        |
|             | 13        | 0   |        |        |        |        |        |
|             | 14        | 0   |        |        |        |        |        |
|             | 15        | -   |        |        |        |        |        |
|             | 16        | 0   |        |        |        |        |        |
|             | 17        | 0   |        |        |        |        |        |
|             | 18        | 0   |        |        |        |        |        |
|             | 19        | 16  |        |        |        |        |        |
|             | 20        | 0   |        |        |        |        |        |
| 21          | 1         | 16, 2i  | 0      | 2, 23i | -      | 1, 17i | -      |
|             | 2         | 12, 2i  | 0      | 0      | 0, 7i  | 0, 15i | 0      |
|             | 3         | 2, 26i  | 0      | 0      | 5, 12i | 0, 5i  | -      |
|             | 4         | 0   | 0, 26i | 0      | 0, 1i  | 0      | 0      |
|             | 5         | 0   | 0      | 0      | 0      | 0      | 0      |
|             | 6         | 0   | 0      | 0      | 0      | 0, 25i | 17, 4i |
|             | 7         | 0   | 0      | 0      | -      | 0      | 0      |
|             | 8         | 1, 14i  | 0      | 0      | 0      | 0      | 0      |
|             | 9         | 0   | 1, 31i | 0      | 0, 21i | 0, 21i | 0      |
|             | 10        | 0   | 0      | 0      | -      | 0      | -      |
|             | 11        | 21, 3i  |        |        |        |        |        |
|             | 12        | 0, 18i  |        |        |        |        |        |
|             | 13        | 0, 19i  |        |        |        |        |        |
|             | 14        | 0   |        |        |        |        |        |
|             | 15        | -   |        |        |        |        |        |
|             | 16        | 0   |        |        |        |        |        |
|             | 17        | 0   |        |        |        |        |        |
|             | 18        | 0   |        |        |        |        |        |
|             | 19        | 0, 1i   |        |        |        |        |        |
|             | 20        | 3, 28i  |        |        |        |        |        |

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+/- : dead parental daphnid  
E: number of aborted eggs  
i: number of immobile offspring

## APPENDIX 2 EC VALUES: REPRODUCTION

Table 15 Parameters of the Probit analysis: reproduction

| Parameter                                 | Value    |
|---|----------|
| Computation runs:                         | 9.0000   |
| Slope b:                                  | 2.6865   |
| Intercept a:                              | -6.23209 |
| Variance of b:                            | 10.0314  |
| Goodness of Fit                           |          |
| Chi <sup>2</sup> :                        | 0.1278   |
| Degrees of freedom:                       | 3.0000   |
| p(Chi <sup>2</sup> ):                     | 0.9883   |
| Log EC50:                                 | 2.3197   |
| SE Log EC50:                              | 0.4837   |
| g-Criterion:                              | 0.5997   |
| Residual Variance (Chi <sup>2</sup> /df): | 0.0426   |
| r <sup>2</sup> :                          | 0.8490   |
| F:  | 16.8850  |
| p(F) (df: 1;3):                           | 0.0260   |

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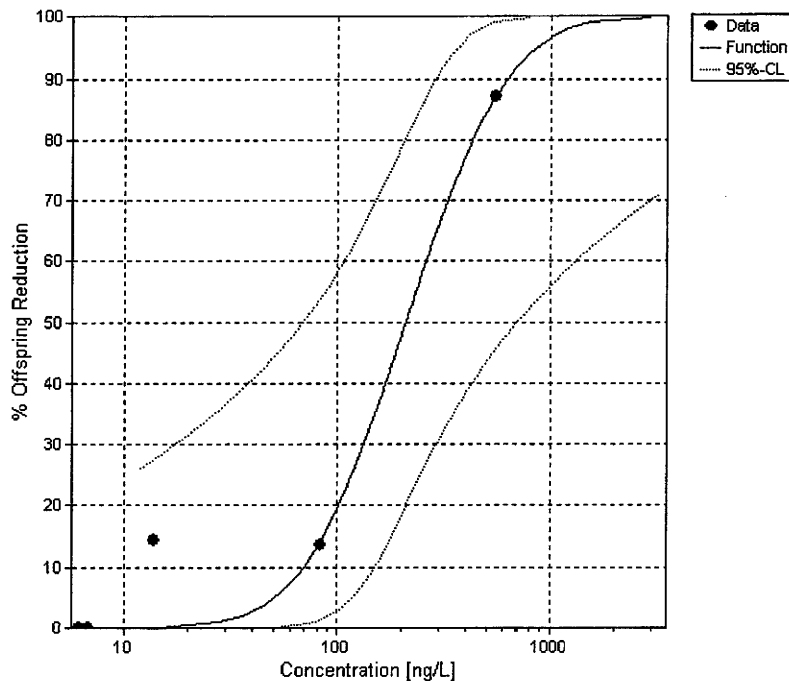


Figure 2 Concentration-effect curve showing the influence of the test item on mean cumulative offspring per survivor of the introduced *Daphnia magna* at 21 day of exposure

Table 16 Results of the Probit analysis: reproduction

| Parameter    | EC10  | EC50  |
|--------------|-------|-------|
| Value (ng/l) | 69.6  | 208.8 |
| lower 95%-cl | 1.3   | 69.0  |
| upper 95%-cl | 149.9 | 698.1 |

**APPENDIX 3 STATISTICS: REPRODUCTION****Normal distribution****Shapiro-Wilk's Test on Normal Distribution**

| Treatm.<br>[% WSF prepared at 5.0 mg/l] | Mean | s  | n  |
|---|------|----|----|
| Control                                 | 84   | 13 | 19 |
| 0.2                                     | 87   | 10 | 10 |
| 0.8                                     | 88   | 17 | 10 |
| 4.0                                     | 72   | 15 | 7  |
| 20.0                                    | 73   | 15 | 10 |
| 100.0                                   | 11   | 06 | 7  |

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Results:

Number of residues = 56

Shapiro-Wilk's W = 0.990

p(W) = 0.909

p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed ( $p > 0.05$ ).

**Homogeneity of variance****Levene's Test on Variance Homogeneity (with Residuals)**

| Source    | SS      | df | MSS   | F     | p(F)  |
|-----------|---------|----|-------|-------|-------|
| Treatment | 288328  | 5  | 57666 | 1.245 | 0.300 |
| Residuals | 2639056 | 57 | 46299 |       |       |
| Total     | 2927384 | 62 |       |       |       |

Based on the pre-selected significance level of 0.05, the Levene test indicates variance homogeneity

Variance homogeneity check passed.

Normal distribution and variance homogeneity requirements are fulfilled.

A parametric multiple test is advisable.

**Determination of NOEC****Williams Multiple Sequential t-test Procedure**

| Treatm.<br>[% WSF prepared at 5.0 mg/l] | Mean | s  | df | LhM | %MDD | t      | t*    | Sign. |
|---|------|----|----|-----|------|--------|-------|-------|
| Control                                 | 84   | 13 |    |     |      |        |       |       |
| 0.2                                     | 87   | 13 | 57 | 87  | -10  | 0.64   | -1.67 | -     |
| 0.8                                     | 88   | 13 | 57 | 87  | -11  | 0.64   | -1.73 | -     |
| 4.0                                     | 72   | 13 | 57 | 72  | -12  | -2.02  | -1.75 | +     |
| 20.0                                    | 73   | 13 | 57 | 72  | -11  | -2.28  | -1.76 | +     |
| 100.0                                   | 11   | 13 | 57 | 11  | -12  | -12.59 | -1.76 | +     |

+: significant; -: non-significant

A NOEC of 0.8 % WSF prepared at 5.0 mg/l is suggested by the program.

**APPENDIX 4 STATISTICS: MORTALITY OF PARENTAL DAPHNIDS****Fisher's Exact Binomial Test with Bonferroni Correction**

| Treatm.<br>[% WSF prepared at 5.0<br>mg/l] | Introduced | Mobile | Immobile | % Immobility | p     | alpha* | sign. |
|--|------------|--------|----------|--------------|-------|--------|-------|
| Control                                    | 20         | 19     | 1        | 5.0          |       |        |       |
| 0.2  | 10         | 10     | 0        | 0.0          | 1.000 | 0.050  | -     |
| 0.8  | 10         | 10     | 0        | 0.0          | 1.000 | 0.025  | -     |
| 4.0  | 10         | 7      | 3        | 30.0         | 0.095 | 0.013  | -     |
| 20.0                                       | 10         | 10     | 0        | 0.0          | 1.000 | 0.017  | -     |
| 100.0                                      | 10         | 7      | 3        | 30.0         | 0.095 | 0.010  | -     |

+: significant; -: non-significant

A NOEC of 100% WSF prepared at 5.0 mg/l is suggested by the program.

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## APPENDIX 5 PARENTAL BODY LENGTH

Table 17 Individual values for body length of the surviving parental daphnids

| Replicate | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |      |      |      |      |      |
|-----------|---|------|------|------|------|------|
|           | Control   | 6.2  | 6.8  | 14   | 83   | 550  |
| 1         | 4.39  | 3.95 | 4.34 | n.d  | 4.24 | n.d  |
| 2         | 4.00  | 4.24 | 3.95 | 4.29 | 4.39 | 3.61 |
| 3         | 4.15  | 4.20 | 4.05 | 4.10 | 4.24 | n.d  |
| 4         | 4.00  | 4.05 | 4.20 | 4.15 | 4.34 | 3.66 |
| 5         | 4.34  | 4.15 | 3.95 | 4.10 | 4.39 | 3.90 |
| 6         | 4.39  | 4.15 | 4.05 | 3.95 | 4.34 | 3.71 |
| 7         | 3.95  | 4.29 | 4.24 | n.d  | 3.95 | 3.46 |
| 8         | 4.00  | 4.29 | 3.90 | 4.39 | 4.29 | 3.80 |
| 9         | 4.34  | 4.29 | 3.95 | 4.10 | 4.15 | 3.66 |
| 10        | 4.34  | 4.00 | 4.39 | n.d  | 4.24 | n.d  |
| 11        | 4.39  |      |      |      |      |      |
| 12        | 4.20  |      |      |      |      |      |
| 13        | 4.00  |      |      |      |      |      |
| 14        | 3.95  |      |      |      |      |      |
| 15        | n.d   |      |      |      |      |      |
| 16        | 4.34  |      |      |      |      |      |
| 17        | 4.10  |      |      |      |      |      |
| 18        | 4.10  |      |      |      |      |      |
| 19        | 4.29  |      |      |      |      |      |
| 20        | 4.15  |      |      |      |      |      |
| Mean:     | 4.18  | 4.16 | 4.10 | 4.15 | 4.26 | 3.69 |
| Std.Dev.: | 0.17  | 0.13 | 0.18 | 0.15 | 0.13 | 0.14 |
| n:        | 19  | 10   | 10   | 7    | 10   | 7    |
| CV:       | 4.0   | 3.0  | 4.3  | 3.5  | 3.1  | 3.8  |

n.d. not determined, parental daphnid did not survive exposure

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**APPENDIX 6 STATISTICS: BODY LENGTH****Shapiro-Wilk's Test on Normal Distribution**

| Treatm.<br>[% WSF prepared at 5.0 mg/l] | Mean | s     | n  |
|---|------|-------|----|
| Control                                 | 4.18 | 0.167 | 19 |
| 0.2                                     | 4.16 | 0.126 | 10 |
| 0.8                                     | 4.10 | 0.177 | 10 |
| 4.0                                     | 4.15 | 0.145 | 7  |
| 20.0                                    | 4.26 | 0.132 | 10 |
| 100.0                                   | 3.69 | 0.140 | 7  |

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Results:

Number of residues = 34

Shapiro-Wilk's W = 0.967

p(W) = 0.391

p(W) is greater than the selected significance level of 0.05;  
therefore, treatment data do  
not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).

**Levene's Test on Variance Homogeneity (with Residuals)**

| Source    | SS     | df | MSS    | F     | p(F)  |
|-----------|--------|----|--------|-------|-------|
| Treatment | 0.0020 | 5  | 0.0000 | 0.868 | 0.508 |
| Residuals | 0.0260 | 57 | 0.0000 |       |       |
| Total     | 0.0280 | 62 |        |       |       |

Based on the pre-selected significance level of 0.05, the Levene test indicates variance homogeneity

Variance homogeneity check passed.

Normal distribution and variance homogeneity requirements are fulfilled.

A parametric multiple test is advisable.

**Williams Multiple Sequential t-test Procedure**

| Treatm.<br>[% WSF prepared at 5.0 mg/l] | Mean | s     | df | LhM  | %MDD  | t     | t*    | Sign. |
|---|------|-------|----|------|-------|-------|-------|-------|
| Control                                 | 4.18 | 0.153 |    |      |       |       |       |       |
| 0.2                                     | 4.16 | 0.153 | 57 | 4.16 | -2.39 | -0.31 | -1.67 | -     |
| 0.8                                     | 4.10 | 0.153 | 57 | 4.17 | -2.47 | -0.11 | -1.73 | -     |
| 4.0                                     | 4.15 | 0.153 | 57 | 4.17 | -2.82 | -0.09 | -1.75 | -     |
| 20.0                                    | 4.26 | 0.153 | 57 | 4.17 | -2.51 | -0.11 | -1.76 | -     |
| 100.0                                   | 3.69 | 0.153 | 57 | 3.69 | -2.84 | -7.31 | -1.76 | +     |

+: significant; -: non-significant

A NOEC of 20.0 % WSF prepared at 5.0 mg/l is suggested by the program.

## APPENDIX 7 EXPERIMENTAL CONDITIONS

**Table 18** pH values measured at various days during the test in freshly prepared media (F) and 48-hour old media (O)

| Nominal day |   | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |     |     |     |     |     |
|-------------|---|---|-----|-----|-----|-----|-----|
|             |   | Control   | 6.2 | 6.8 | 14  | 83  | 550 |
| 0           | F | 8.0   | 8.0 | 8.0 | 8.0 | 8.0 | 8.0 |
| 2           | O | 8.8   | 8.9 | 8.9 | 9.0 | 8.9 | 8.9 |
|             | F | 8.0   | 8.0 | 8.0 | 8.0 | 8.0 | 8.0 |
| 4           | O | 8.4   | 8.7 | 8.7 | 8.7 | 8.7 | 8.8 |
|             | F | 8.0   | 8.1 | 8.1 | 8.0 | 8.0 | 8.0 |
| 6           | O | 8.5   | 8.5 | 8.5 | 8.5 | 8.5 | 8.6 |
|             | F | 8.0   | 8.1 | 8.1 | 8.1 | 8.1 | 8.1 |
| 8           | O | 8.3   | 8.3 | 8.4 | 8.4 | 8.4 | 8.4 |
|             | F | 8.0   | 8.2 | 8.2 | 8.1 | 8.1 | 8.1 |
| 10          | O | 8.3   | 8.4 | 8.4 | 8.4 | 8.4 | 8.5 |
|             | F | 8.0   | 8.0 | 8.0 | 8.0 | 8.0 | 7.9 |
| 12          | O | 8.0   | 8.0 | 8.1 | 8.1 | 8.1 | 8.3 |
|             | F | 7.8   | 7.8 | 7.8 | 7.8 | 7.8 | 7.8 |
| 14          | O | 8.2   | 8.2 | 8.2 | 8.2 | 8.2 | 8.4 |
|             | F | 8.0   | 8.0 | 8.0 | 8.0 | 8.0 | 7.9 |
| 16          | O | 8.2   | 8.1 | 8.2 | 8.3 | 8.2 | 8.5 |
|             | F | 8.0   | 8.0 | 7.9 | 7.9 | 7.9 | 7.9 |
| 18          | O | 8.2   | 8.3 | 8.2 | 8.3 | 8.3 | 8.3 |
|             | F | 7.9   | 8.0 | 8.0 | 8.0 | 8.0 | 8.0 |
| 20          | O | 8.2   | 8.3 | 8.3 | 8.3 | 8.3 | 8.4 |
|             | F | 8.0   | 8.0 | 8.0 | 8.0 | 8.0 | 7.9 |
| 21          | O | 8.2   | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 |

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**Table 19** Dissolved oxygen concentrations (mg/l) measured at various days during the test in freshly prepared media (F) and 48-hour old media (O)

| Nominal day |   | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |     |     |     |     |     |
|-------------|---|---|-----|-----|-----|-----|-----|
|             |   | Control   | 6.2 | 6.8 | 14  | 83  | 550 |
| 0           | F | 9.2   | 9.2 | 9.2 | 9.2 | 9.2 | 9.0 |
| 2           | O | 11  | 11  | 11  | 11  | 11  | 11  |
|             | F | 9.3   | 9.3 | 9.4 | 9.3 | 9.2 | 9.0 |
| 4           | O | 9.8   | 10  | 10  | 10  | 10  | 10  |
|             | F | 8.9   | 9.0 | 9.0 | 9.0 | 9.0 | 8.9 |
| 6           | O | 9.7   | 9.8 | 9.9 | 9.6 | 9.8 | 10  |
|             | F | 8.9   | 8.8 | 8.8 | 8.7 | 8.8 | 8.7 |
| 8           | O | 9.4   | 9.6 | 9.6 | 9.7 | 9.6 | 9.6 |
|             | F | 9.0   | 9.1 | 9.0 | 8.9 | 8.9 | 8.9 |
| 10          | O | 9.3   | 9.5 | 9.5 | 9.7 | 9.7 | 10  |
|             | F | 9.1   | 9.1 | 9.1 | 9.1 | 9.1 | 8.9 |
| 12          | O | 9.8   | 10  | 10  | 10  | 10  | 11  |
|             | F | 9.6   | 9.5 | 9.6 | 9.6 | 9.6 | 9.2 |
| 14          | O | 9.5   | 9.5 | 9.6 | 9.8 | 9.6 | 10  |
|             | F | 9.1   | 9.1 | 9.1 | 9.1 | 9.0 | 8.8 |
| 16          | O | 9.3   | 9.4 | 9.3 | 9.6 | 9.6 | 10  |
|             | F | 9.1   | 9.1 | 9.0 | 9.1 | 9.1 | 8.9 |
| 18          | O | 9.3   | 9.3 | 9.4 | 9.7 | 9.5 | 9.7 |
|             | F | 9.2   | 9.1 | 9.1 | 9.1 | 9.1 | 8.8 |
| 20          | O | 9.8   | 9.8 | 9.9 | 10  | 9.9 | 9.7 |
|             | F | 9.2   | 9.3 | 9.3 | 9.3 | 9.2 | 9.1 |
| 21          | O | 9.6   | 9.5 | 9.6 | 9.7 | 9.6 | 9.7 |

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**Table 20** Temperature (°C) measured at various days during the test in freshly prepared media (F) and 48-hour old media (O)

| Nominal day |   | 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline), concentration (ng/l) |     |     |    |    |     |
|-------------|---|---|-----|-----|----|----|-----|
|             |   | Control   | 6.2 | 6.8 | 14 | 83 | 550 |
| 0           | F | 20  | 20  | 20  | 20 | 20 | 21  |
| 2           | O | 20  | 20  | 20  | 20 | 20 | 20  |
|             | F | 19  | 19  | 19  | 19 | 20 | 21  |
| 4           | O | 20  | 20  | 20  | 20 | 20 | 20  |
|             | F | 20  | 20  | 20  | 20 | 21 | 21  |
| 6           | O | 20  | 20  | 20  | 20 | 20 | 20  |
|             | F | 20  | 20  | 20  | 20 | 20 | 20  |
| 8           | O | 20  | 20  | 20  | 20 | 20 | 20  |
|             | F | 19  | 19  | 19  | 20 | 20 | 20  |
| 10          | O | 19  | 20  | 20  | 20 | 20 | 20  |
|             | F | 20  | 20  | 20  | 20 | 20 | 21  |
| 12          | O | 20  | 20  | 20  | 20 | 20 | 20  |
|             | F | 19  | 20  | 20  | 20 | 20 | 21  |
| 14          | O | 20  | 20  | 20  | 20 | 20 | 20  |
|             | F | 20  | 20  | 20  | 20 | 20 | 21  |
| 16          | O | 20  | 20  | 20  | 20 | 20 | 20  |
|             | F | 19  | 20  | 20  | 20 | 20 | 20  |
| 18          | O | 20  | 20  | 20  | 20 | 20 | 20  |
|             | F | 19  | 19  | 19  | 20 | 20 | 20  |
| 20          | O | 19  | 19  | 20  | 20 | 20 | 20  |
|             | F | 19  | 19  | 19  | 19 | 19 | 20  |
| 21          | O | 19  | 20  | 20  | 20 | 20 | 20  |

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**Table 21** Hardness (in mg/l CaCO<sub>3</sub>) measured at various days during the test in freshly prepared media (F) and 48-hour old media (O)

| Nominal day |   | Test substance <sup>1</sup> , concentration (ng/l) |     |
|-------------|---|--|-----|
|             |   | Control  | 550 |
| 4           | F | 214  | 214 |
|             | O | 214  | 214 |
| 10          | F | 196  | 196 |
|             | O | 196  | 196 |
| 18          | F | 196  | 196 |
|             | O | 196  | 179 |

1. 4,4'-(9H-fluoren-9-ylidene)bis(2-chloroaniline)

**APPENDIX 8 ANALYTICAL REPORT**

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## **DETERMINATION OF THE CONCENTRATIONS**

Author

K.A. Oudhoff, PhD.

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## 2. REPORT APPROVAL

WIL Research Europe B.V.

Principal Scientist  
Analytical Chemistry

K.A. Oudhoff, PhD.

A handwritten signature in black ink, appearing to read 'K.A. Oudhoff', is written over a horizontal line.

Date: ...20... May ...2014.....

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### 3. INTRODUCTION

#### 3.1. Preface

Study plan analytical phase

Start : 05 November 2013

Completion : 17 April 2014

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#### 3.2. Aim of the study

The purpose of the analytical phase was to determine the actual concentrations in samples taken from the test solutions used during the ecotoxicity test.

### 4. MATERIALS AND METHODS

#### 4.1. Reagents

|              |   |
|--------------|---|
| Water        | Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA) |
| Acetonitrile | Biosolve, Valkenswaard, The Netherlands   |
| Formic acid  | Biosolve  |
| M7-medium    | See main report   |

All reagents were of analytical grade, unless specified otherwise.

#### 4.2. Samples

The test samples were stored in the freezer ( $\leq -15^{\circ}\text{C}$ ). Storage stability of samples under these conditions were demonstrated in project 503717 and project 504759.

On the day of analysis, the test samples were defrosted at room temperature. The samples were diluted in a 1:1 (v:v) ratio with acetonitrile and analysed. If necessary, the samples were further diluted with 50/50 (v/v) acetonitrile/M7-medium to obtain concentrations within the calibration range.

#### 4.3. Analytical method

##### 4.3.1. Analytical conditions

Quantitative analysis was based on the analytical methods validated for the test substance in projects 503717 (HPLC-UV) and 504759 (UPLC-MS/MS).

##### HPLC-UV applied in the range-finding test

|                    |   |
|--------------------|---|
| Instrument         | Alliance Separation Module 2695 (Waters, Milford, MA, USA)                      |
| Detector           | Dual $\lambda$ Absorbance Detector 2487 (Waters)                                |
| Column             | Symmetry Shield RP-18, 150 mm $\times$ 3 mm i.d., dp = 5 $\mu\text{m}$ (Waters) |
| Column temperature | $40^{\circ}\text{C} \pm 1^{\circ}\text{C}$                                      |
| Injection volume   | 50 $\mu\text{l}$  |
| Mobile phase       | 70/30 (v/v) acetonitrile/water  |
| Flow               | 1 ml/min  |
| UV detection       | 230 nm  |

UPLC-MS/MS applied in the third reproduction test:

|                    |   |
|--------------------|---|
| Instrument         | Acquity UPLC system (Waters, Milford, MA, USA)                              |
| Detector           | Xevo TQ-S mass spectrometer (Waters)  |
| Column             | Acquity UPLC BEH Shield RP18, 100 mm × 2.1 mm i.d.,<br>dp = 1.7 µm (Waters) |
| Column temperature | 40°C ± 1°C  |
| Injection volume   | 10 µl   |
| Mobile phase       | 0.1% formic acid in 70/30 (v/v) acetonitrile/water                          |
| Flow               | 0.6 ml/min  |

## MS detection

|                   |                                     |
|-------------------|-------------------------------------|
| Ionisation source | ESI <sup>+</sup>                    |
| Cone voltage      | 80 V                                |
| Collision energy  | 34 V                                |
| Quantitation      | <i>m/z</i> 416.9 → <i>m/z</i> 290.0 |

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**4.3.2. Preparation of solutions**Stock and spiking solutions

Stock solutions of the test substance were prepared in acetonitrile at concentrations of 985 - 3430 mg/l. In order to dissolve the test substance the solutions were ultrasonicated for 5 minutes.

Spiking solutions were made up from a stock solution and/or dilutions of this solution. The solvent of the spiking solutions was acetonitrile.

Calibration solutionsRange-finding test

Calibration solutions in the concentration range of 0.004 – 10 mg/l were prepared from two stock solutions. The end solution of the calibration solutions was 50/50 (v/v) acetonitrile/water.

Third reproduction test

Solutions with the test substance in the concentration range of 200 - 5000 ng/l were prepared in acetonitrile from two stock solutions. The solutions were 100-times diluted with 50/50 (v/v) acetonitrile / M7-medium to obtain calibration solutions in the concentration range of 2 - 50 ng/l.

Procedural recovery samples

2 ml blank medium was spiked with the test substance at a target concentration of 0.1 or 100 mg/l (range-finding test) and 5, 10 or 5000 ng/l (third reproduction test). The accuracy samples were treated similarly as the test samples (see paragraph 4.2 'Samples').

Blank procedural recovery samples were prepared and treated similarly to the test samples.

**4.3.3. Sample injections**

Calibration solutions were injected in duplicate. Test samples and procedural recovery samples were analysed by single injection.

**4.4. Electronic data capture**

System control, data acquisition and data processing were performed using the following programmes:

- Empower version 7.00 (Waters, Milford, MA, USA).
- MassLynx version 4.1 (Waters).

Temperature, relative humidity and/or atmospheric pressure during sample storage and/or performance of the studies was monitored continuously using the following programme:

- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA).

**4.5. Formulas**

Response (R) Peak area test substance [units]

Calibration curve  $R = a C_N + b$

where:

$C_N$  = nominal concentration [mg/l or ng/l]

$a$  = slope [units  $\times$  l/mg or units  $\times$  l/ng]

$b$  = intercept [units]

Analysed concentration ( $C_A$ )

$$C_A = \frac{(R - b)}{a} \times d \quad [\text{mg/l or ng/l}]$$

where:

$d$  = dilution factor

Recovery

$$\frac{C_A}{C_N} \times 100 \quad [\%]$$

Relative to initial

$$\frac{C_A (t = \text{old})}{C_A (t = \text{fresh})} \times 100 \quad [\%]$$

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## 5. RESULTS

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### 5.1. Calibration curves

#### *Range-finding test*

A calibration curve was constructed using five concentrations. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a  $1/\text{concentration}^2$  weighting factor. The coefficient of correlation ( $r$ ) was  $> 0.99$ .

#### *Third reproduction test*

Calibration curves were constructed using six concentrations. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a  $1/\text{concentration}^2$  weighting factor. Two of twelve responses were excluded from the curves since the back calculated accuracy was  $> 15\%$  from the nominal concentration. The coefficient of correlation ( $r$ ) was  $> 0.99$  for each curve.

### 5.2. Samples

#### 5.2.1. Range-finding test

The results for the procedural recovery samples and test samples of the range-finding test are given in Table 1 and Table 2.

The chromatograms of the blank procedural recovery samples showed no peak at the retention time of the test substance. It demonstrated that the sample treatment was adequate for the test samples.

The mean recoveries of the procedural recovery samples containing test substance fell within the criterion of 70-110%. It demonstrated that the analytical method was adequate for the determination of the test substance concentration in the test samples.

#### 5.2.2. Third reproduction test

The results for the procedural recovery samples and test samples of the third reproduction test are given in Table 3 and Table 4.

Relatively small background responses of the test substance were detected in the chromatograms of the blank samples and procedural recovery samples at 5 ng/l (results are archived in the raw data). The background was a result of the requirement to detect the compound at very low concentration levels. The impact of the background was minimal for samples at concentrations of 10 ng/l and higher. The LOQ level of the method was assessed at this level for analysis of the samples of the third reproduction test (15 to 17 April 2014).

The mean recoveries of the procedural recovery samples containing test substance were between 100% and 120%. The results were accepted. The method was used for the determination of the test substance at very low concentration levels. Variation on the response is therefore somewhat higher than general by analysis. The MS method was considered to be best possible for the determination of the test substance in aqueous samples in order to support the ecotoxicological studies. Matrix-matched calibration standards were applied to minimize the effect of the salt medium to the response of the test substance.

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## TABLES

**Table 1 Procedural recovery samples - Range-finding test**

| Date of preparation | Date of analysis | Target concentration [mg/l] | Nominal concentration [mg/l] | Analysed concentration [mg/l] | Recovery [%] | Mean recovery [%] |
|---------------------|------------------|-----------------------------|------------------------------|-------------------------------|--------------|-------------------|
| 05-Nov-2013         | 05-Nov-2013      | 0                           | 0<br>0                       | n.d.<br>n.d.                  | n.a.<br>n.a. | n.a.              |
| 05-Nov-2013         | 05-Nov-2013      | 0.1                         | 0.0995<br>0.0995             | 0.0982<br>0.0990              | 99<br>99     | 99                |
| 05-Nov-2013         | 05-Nov-2013      | 100                         | 99.5<br>99.5                 | 108<br>108                    | 108<br>109   | 109               |

n.d. Not detected.

n.a. Not applicable.

**Table 2 Concentrations of the test substance in test medium - Range-finding test**

| Time of sampling [days] | Date of sampling | Date of analysis <sup>1</sup> | Percentage of WSF <sup>2</sup> [%]             | Analysed concentration [mg/l]   | Relative to initial [%]    |
|-------------------------|------------------|-------------------------------|--|---|----------------------------|
| 0<br>(fresh)            | 29-Oct-2013      | 05-Nov-2013                   | 0<br>100                                       | n.d.<br>0.00178 <sup>4</sup>  |                            |
| 2<br>(old)              | 31-Oct-2013      | 05-Nov-2013                   | 0<br>100<br>0 <sup>3</sup><br>100 <sup>3</sup> | n.d.<br>0.00195 <sup>4</sup><br>n.d.<br>0.00125 <sup>4</sup>                | n.a.<br>110<br>n.a.<br>70  |
| 2<br>(fresh)            | 31-Oct-2013      | 05-Nov-2013                   | 0<br>100                                       | n.d.<br>0.00100 <sup>4</sup>  |                            |
| 3<br>(old)              | 01-Nov-2013      | 05-Nov-2013                   | 0<br>100<br>0 <sup>3</sup><br>100 <sup>3</sup> | n.d.<br>0.000847 <sup>4</sup><br>n.d. <sup>3</sup><br>0.000806 <sup>4</sup> | n.a.<br>85<br>n.a.<br>80   |
| 3<br>(fresh)            | 01-Nov-2013      | 05-Nov-2013                   | 0<br>100                                       | n.d.<br>0.00103 <sup>4</sup>  |                            |
| 6<br>(old)              | 04-Nov-2013      | 05-Nov-2013                   | 0<br>100<br>0 <sup>3</sup><br>100 <sup>3</sup> | n.d.<br>n.d.<br>n.d.<br>0.000561 <sup>4</sup>                               | n.a.<br>n.a.<br>n.a.<br>54 |

<sup>1</sup> Samples were stored in the freezer ( $\leq -15^{\circ}\text{C}$ ) until the day of analysis.<sup>2</sup> Percentage of a water soluble fraction (WSF) prepared at a loading rate of 100 mg/l.<sup>3</sup> Without daphnia's and algae.<sup>4</sup> Estimated value, calculated using the mean response factor of the lowest calibration solution.

n.d. Not detected.

n.a. Not applicable.

**Table 3 Procedural recovery samples - Third reproduction test**

| Date of preparation | Date of analysis | Target concentration [ng/l] | Nominal concentration [ng/l] | Analysed concentration [ng/l] | Recovery [%] | Mean recovery [%] |
|---------------------|------------------|-----------------------------|------------------------------|-------------------------------|--------------|-------------------|
| 15-Apr-2014         | 15-Apr-2014      | 0                           | 0<br>0                       | < LOQ<br>< LOQ                | n.a.<br>n.a. | n.a.              |
| 15-Apr-2014         | 15-Apr-2014      | 10                          | 10.0<br>10.0                 | 11.5<br>8.54                  | 115<br>85    | 100               |
| 15-Apr-2014         | 15-Apr-2014      | 5000                        | 5000<br>5000                 | 5884<br>6072                  | 118<br>121   | 120               |
| 17-Apr-2014         | 17-Apr-2014      | 0                           | 0<br>0                       | < LOQ<br>< LOQ                | n.a.<br>n.a. | n.a.              |
| 17-Apr-2014         | 17-Apr-2014      | 10                          | 10.0<br>10.0                 | 12.0<br>11.2                  | 120<br>112   | 116               |
| 17-Apr-2014         | 17-Apr-2014      | 5000                        | 5000<br>5000                 | 5306<br>5346                  | 106<br>107   | 107               |

LOQ The limit of quantification (LOQ) was assessed at 10 ng/l.

n.a. Not applicable.

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**Table 4 Concentrations of the test substance in test medium - Third reproduction test**

| Time of sampling [days] | Date of sampling | Date of analysis <sup>1</sup> | Percentage of WSF <sup>2</sup> [%] | Analysed concentration [ng/l] | Relative to initial [%] |
|-------------------------|------------------|-------------------------------|------------------------------------|-------------------------------|-------------------------|
| 0<br>(fresh)            | 21-Mar-2014      | 15-Apr-2014                   | 0                                  | < LOQ                         |                         |
|                         |                  | 15-Apr-2014                   | 0.16                               | 18.2                          |                         |
|                         |                  | 17-Apr-2014                   | 0.16 <sup>3</sup>                  | < LOQ                         |                         |
|                         |                  | 15-Apr-2014                   | 0.80                               | < LOQ                         |                         |
|                         |                  | 17-Apr-2014                   | 0.80 <sup>3</sup>                  | < LOQ                         |                         |
|                         |                  | 15-Apr-2014                   | 4.0                                | 33.7                          |                         |
|                         |                  | 15-Apr-2014                   | 20                                 | 158                           |                         |
|                         |                  | 15-Apr-2014                   | 100                                | 926                           |                         |
| 2<br>(old)              | 23-Mar-2014      | 15-Apr-2014                   | 0                                  | 26.9                          | n.a.                    |
|                         |                  | 17-Apr-2014                   | 0 <sup>3</sup>                     | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 0.16                               | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 0.80                               | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 4.0                                | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 20                                 | 94.6                          | 60                      |
|                         |                  | 16-Apr-2014                   | 100                                | 228                           | 25                      |
|                         |                  |                               |                                    |                               |                         |
| 4<br>(fresh)            | 25-Mar-2014      | 15-Apr-2014                   | 0                                  | < LOQ                         |                         |
|                         |                  | 15-Apr-2014                   | 0.16                               | < LOQ                         |                         |
|                         |                  | 15-Apr-2014                   | 0.80                               | 19.1                          |                         |
|                         |                  | 15-Apr-2014                   | 4.0                                | 30.1                          |                         |
|                         |                  | 15-Apr-2014                   | 20                                 | 315                           |                         |
|                         |                  | 15-Apr-2014                   | 100                                | 1697                          |                         |

Table 4 Continued

| Time of sampling [days] | Date of sampling | Date of analysis <sup>1</sup> | Percentage of WSF <sup>2</sup> [%] | Analysed concentration [ng/l] | Relative to initial [%] |
|-------------------------|------------------|-------------------------------|------------------------------------|-------------------------------|-------------------------|
| 6 (old)                 | 27-Mar-2014      | 15-Apr-2014                   | 0                                  | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 0.16                               | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 0.80                               | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 4.0                                | < LOQ                         | n.a.                    |
|                         |                  | 16-Apr-2014                   | 20                                 | 21.6                          | 6.8                     |
|                         |                  | 16-Apr-2014                   | 100                                | 223                           | 15                      |
| 12 (fresh)              | 02-Apr-2014      | 15-Apr-2014                   | 0                                  | < LOQ                         |                         |
|                         |                  | 15-Apr-2014                   | 0.16                               | < LOQ                         |                         |
|                         |                  | 15-Apr-2014                   | 0.80                               | < LOQ                         |                         |
|                         |                  | 15-Apr-2014                   | 4.0                                | 39.3                          |                         |
|                         |                  | 15-Apr-2014                   | 20                                 | 209                           |                         |
|                         |                  | 15-Apr-2014                   | 100                                | 1127                          |                         |
| 14 (old)                | 04-Apr-2014      | 15-Apr-2014                   | 0                                  | < LOQ                         | n.a.                    |
|                         |                  | 17-Apr-2014                   | 0.16                               | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 0.80                               | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 4.0                                | < LOQ                         | n.a.                    |
|                         |                  | 16-Apr-2014                   | 20                                 | 18.5                          | 8.9                     |
|                         |                  | 15-Apr-2014                   | 100                                | 442                           | 39                      |
| 18 (fresh)              | 08-Apr-2014      | 15-Apr-2014                   | 0                                  | < LOQ                         |                         |
|                         |                  | 17-Apr-2014                   | 0.16                               | 10.1                          |                         |
|                         |                  | 15-Apr-2014                   | 0.80                               | 10.6                          |                         |
|                         |                  | 15-Apr-2014                   | 4.0                                | 44.1                          |                         |
|                         |                  | 16-Apr-2014                   | 20                                 | 202                           |                         |
|                         |                  | 15-Apr-2014                   | 100                                | 1323                          |                         |
| 20 (old)                | 10-Apr-2014      | 15-Apr-2014                   | 0                                  | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 0.16                               | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 0.80                               | < LOQ                         | n.a.                    |
|                         |                  | 15-Apr-2014                   | 4.0                                | < LOQ                         | n.a.                    |
|                         |                  | 16-Apr-2014                   | 20                                 | 20.5                          | 10                      |
|                         |                  | 16-Apr-2014                   | 100                                | 132                           | 10                      |

<sup>1</sup> Samples were stored in the freezer ( $\leq -15^{\circ}\text{C}$ ) until the day of analysis.

<sup>2</sup> Percentage of a water soluble fraction (WSF) prepared at a loading rate of 5 mg/l.

<sup>3</sup> Reserve sample.

LOQ The limit of quantification (LOQ) was assessed at 10 ng/l based on the results of the procedural recovery samples.

n.a. Not applicable.

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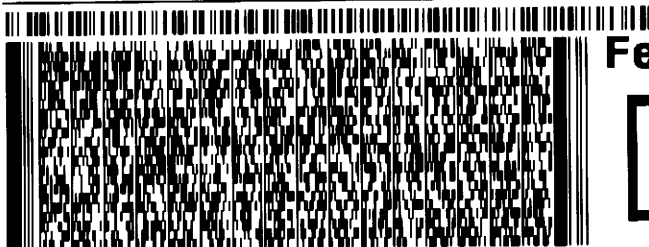
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